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Microcystin-LR induces anoikis resistance to the hepatocyte uptake transporter OATP1B3-expressing cell lines

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ABSTRACT

Microcystin-LR is a cyclic peptide released by several bloom-forming cyanobacteria. Understanding the mechanism of microcystin-LR toxicity is important, because of the both potencies of its acute cytotoxicity and tumor-promoting activity in hepatocytes of animals and humans. Recently, we have reported that the expression of human hepatocyte uptake transporter OATP1B3 was critical for the selective uptake of microcystin-LR into hepatocytes and for induction of its fatal cytotoxicity. In this study, we demonstrated a novel function of microcystin-LR which induced bipotential changes including anoikis resistance and cytoskeleton reorganization to OATP1B3-transfected HEK293 cells (HEK293-OATP1B3). After exposure to microcystin-LR, HEK293-OATP1B3 cells were divided to the floating cells and remaining adherent cells. After collection and reseeding the floating cells into a fresh flask, cells were confluently proliferated (HEK293-OATP1B3-FL) under the microcystin-LR-free condition. Both the proliferated HEK293-OATP1B3-FL and remaining adherent HEK293-OATP1B3-AD cells changed the character with down- and up-regulation of E-cadherin, respectively. Additionally, these cells acquired resistance to microcystin-LR. These results suggest that microcystin-LR could be associated with not only tumor promotion, but also epithelial–mesenchymal transition-mediated cancer metastasis. Furthermore, microcystin-LR might induce the cytoskeleton reorganization be accompanied epithelial–mesenchymal transition.

1. Introduction

Hepatic tumor promoter microcystin-LR is a cyclic heptapeptide released by several bloom-forming toxic cyanobacteria (Carmichael and Falconer, 1993; Dietrich and Hoeger, 2005; Gehringer, 2004). Microcystin-LR toxicity is primarily caused by inhibition of the serine/threonine protein phosphatases, PP1 and PP2A. Inhibition of the phosphatases induces accumulation of phospho-protein in the hepatocytes, consequently, intracellular signal transductions are disrupted (Komatsu et al., 2007; Takumi et al., 2010). Several PP1 and PP2A inhibitors such as okadaic acid, nodularin, and microcystin-LR are classified as tumor promoters (Fujiki and Suganuma, 1999). A number of epidemiological studies have indicated that consumption of cyanobacterially contaminated water can favor the growth of human hepatocellular carcinoma (Falconer and Humpage, 2005; Ueno et al., 1996; Yuan et al., 2006). On the other hand, microcystin-LR possesses the acute cytotoxicity to the hepatocyte and induces liver injury (Dietrich and Hoeger, 2005; Gehringer, 2004). Investigation to figure out the onset mechanisms of biological activity of microcystin-LR is important for understanding the regulation of cell fate.

It has been known that microcystin-LR has poor cell membrane permeability except for the membranes of hepatocytes (Chong et al., 2000; Eriksson et al., 1990; Nong et al., 2007;

Abbreviations: AD, adherent; CCK-8, cell counting kit-8; EMT, epithelial mesenchymal transition; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; FL, floating; MC-LR, microcystin-LR; IC_{50}, half maximal inhibitory concentration; MEM, minimum essential medium; MTT, 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OATP, organic anion transporting polypeptide; PI, propidium iodide; PP, protein phosphatase; PVDF, polyvinylidene difluoride; SD, standard deviation; WST, water soluble tetrazolium salts; ZEB, zinc-finger E-box binding homeobox; ZO-1, zonula occludens-1 protein.

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Runnegar et al., 1995). Previously, we (Komatsu et al., 2007) and other groups (Fischer et al., 2005; Monks et al., 2007) reported that the expression of the human hepatocyte uptake transporters OATP1B1 or OATP1B3 were critical for the selective uptake of microcystin-LR into hepatocytes and for induction of its fatal cytotoxicity. The molecular basis of the tumor promoting ability of microcystin-LR is unclear but most likely involves protein phosphatase inhibition leading to hyperphosphorylation of many cellular proteins and consequently destruction of cell-cycle control (Fujiki and Suganuma, 1999; Guy et al., 1992; Messner et al., 2006; Takumi et al., 2010).

We have recently demonstrated that a lethal concentration of microcystin-LR stimulates, not only cellular signal transduction leading to cell death, but also cell survival signals (Takumi et al., 2010). Although the lethal concentration of microcystin-LR, which was obtained from MTT cell survival assay, induced apoptotic cell death, apoptosis population was only approximately 10%. (Komatsu et al., 2007). However, the necrosis was negligible after exposure to microcystin-LR (Komatsu et al., 2007). In this study, we tried to resolve the discrepancy about the biological activity of microcystin-LR using HEK293-OATP1B3 cells.

2. Materials and methods

2.1. Reagents and antibodies

Microcystin-LR was purchased from Alexis (Lausen, Switzerland). Pacritaxel was purchased from Sigma (St. Louis, MO). Cisplatin was purchased from Wako (Tokyo, Japan). Fetal calf serum was obtained from Cancera International (Canada). Minimum essential medium (MEM) was purchased from Sigma (St. Louis, MO). Monoclonal mouse antibody against E-cadherin was purchased from BD Biosciences (San Jose, CA). Polyclonal goat antibody against β-actin was purchased from Santa Cruz (Santa Cruz, CA). Monoclonal mouse antibody against α-tubulin was purchased from Santa Cruz (Santa Cruz, CA). Polyclonal rabbit antibodies against N-cadherin, ZO-1, Na^+/K^+-ATPase, snail, slug, ZEB-1, and β-catenin were purchased from Cell Signaling Technology Japan (Tokyo). Polyclonal rabbit antibody against OATP1B3 was purchased from Santa Cruz (Santa Cruz, CA). Polyclonal rabbit antibody against GAPDH was purchased from Gene Tex (Irvine, CA). Secondary antibodies against mouse IgG and rabbit IgG were purchased from GE healthcare science (Buckinghamshire, United Kingdom), and against goat IgG was purchased from Jackson Immuno Research Laboratories (West Grove, PA). Lipofectamine™ 2000 was purchased from Life technologies (Carlsbad, CA). Alexa Fluor® 488-annexin V and propidium iodide (PI) were purchased from Life Technologies (Carlsbad, CA).

2.2. Cell culture

HEK293 cells were stably transfected with the plasmids pcDNA3.1-*SLCO1B3* (HEK293-OATP1B3 cells) or pcDNA3.1-control vector (HEK293-CV cells), which were previously generated (König et al., 2000; Letschert et al., 2004). Before experiments, HEK293-OATP1B3 cells were selected by subcloning twice. Cells were cultured in MEM supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin (MEM-10% FCS) and 400 μg/ml G418 at 37 °C, 100% humidity, and 5% CO_2.

2.3. Anoikis resistance assay

Lethal concentrations, approximately 5-times higher concentrations to IC_{50}-values, of microcystin-LR (50 nM) or paclitaxel (450 nM), or approximately 30-times higher concentration to IC_{50}-value of cisplatin (100 μM) was exposed to HEK293-OATP1B3 cells, and these cells were incubated in the CO_2 incubator for 72 h. After incubation with reagents, the detached floating cells were collected into centrifugation tubes and spun at 1000 × *g* for 3 min at room temperature. The pellet cells were treated with Alexa Fluor® 488-annexin V for 5 min in dark room, and then the cells were treated with PI for 1 min in the dark room. The Alexa Fluor® 488-annexin V and PI treated cells were analysed cell death using image-based cytometer (Tali, Life technologies, Carlsbad, CA).

Next, we investigated viability of the floating cells. Lethal concentrations of microcystin-LR (50 nM) or paclitaxel (450 nM) was exposed to HEK293-OATP1B3 cells, and these cells were incubated in the CO_2 incubator for 72 h. After incubation with reagents, the detached floating cells were collected into centrifugation tubes and spun at 1000 × *g* for 3 min at room temperature. The pellets were re-suspended with PBS and centrifuged again. The washed pellets were re-suspended with MEM-10% FCS, and seeded to fresh flask. Then, these cells were incubated in the CO_2 incubator for another 10 days (HEK293-OATP1B3-FL cells; Fig. 1). On the

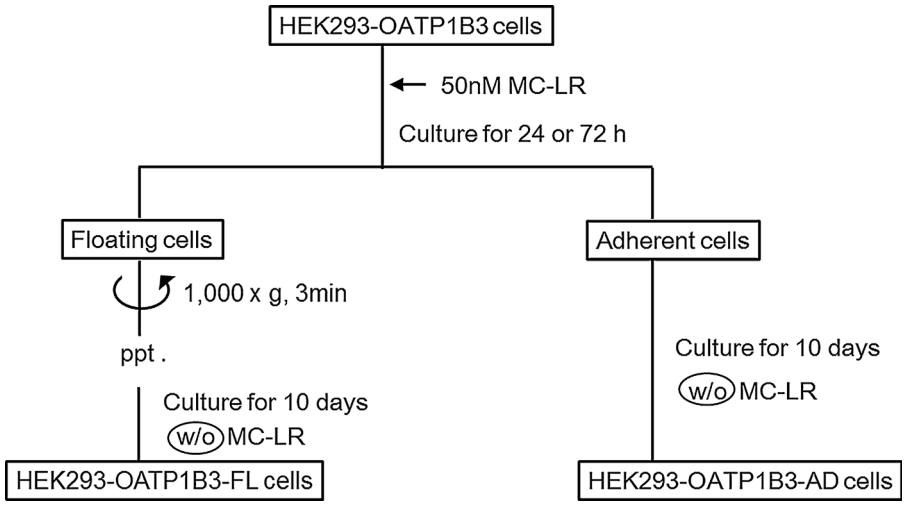

Fig. 1. Scheme for generation of floating cells, HEK293-OATP1B3-FL cells, adherent cells, and HEK293-OATP1B3-AD cells. These cells were prepared after exposure to microcystin-LR as described in Section 2.